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Androstenedione may organize or activate sex-reversed traits in female spotted hyenas

(androgens/sexual differentiation/virilization/aggression/behavioral development)

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ABSTRACT **Spotted hyenas (*Crocuta crocuta* Erxleben) present a unique syndrome of reversal in behavioral and anatomical distinction between the sexes: females are heavier and more aggressive than males and dominant over them. The female's external genitalia include a false scrotum and a fully erectile pseudopenis through which mating and birth take place. Results of studies of circulating testosterone levels in wild spotted hyenas do not account for the "male-like" characteristics of the female. Androstenedione, however, is consistently higher in females than in males, particularly during early infancy. Experiments on rodents show that androstenedione can be a potent organizer of anatomical and behavioral differentiation. This study suggests that it may also produce the profound virilization of female spotted hyenas.**

The behavioral and morphological characteristics of the spotted hyena (*Crocuta crocuta* Erxleben) present an intriguing set of evolutionary and physiological problems. Within a group ("clan") of these nocturnal social hunters, all adult females are dominant to nearly all adult males in most social interactions (1), including access to food (2). Correlated with their marked social dominance, female spotted hyenas are somewhat heavier than males and exhibit extreme masculinization of the external genitalia. The vaginal labia are fused to form a scrotum, whereas the clitoris is developed as a pseudopenis, traversed by the urogenital canal through which the female urinates, copulates, and gives birth. There are no "normal" female external genitalia (3, 4).

The clitoris is similar in size to the male penis and is fully erectile. From infancy through adulthood, both sexes routinely exhibit phallic erections as part of various social displays, including frequent "greetings" during which pairs or groups of hyenas sniff and lick each others' genital regions (1). Gould (5) suggested that the anatomical virilization of female spotted hyenas is an unselected secondary result of behavioral virilization, mediated by elevated prenatal androgen levels, that evolved in response to some aspect of the species' social behavior. Hamilton *et al.* (6) proposed that this selective pressure was competition with males over access to carcasses. Subsequently, Frank (2) found that the ability of young spotted hyenas to feed among adults at a kill is correlated with their mothers' social rank. He suggested that female dominance and the associated masculinization may have evolved in response to the selective pressures of offspring survival in a species in which feeding is exceptionally competitive.

Testosterone has commonly been associated with aggression and dominance in mammals, either as a differentiating hormone (or prohormone) during a critical phase of development or as an activating agent in prepubertal or adult animals (7). Androgen production by the fetal testis also is responsible for differentiation of the male external genitalia, whereas postnatal androgens facilitate genital growth, primarily at puberty (8). Because of these links between androgens (particularly testosterone), social behavior, and morphology, several investigators have examined plasma androgen levels in female spotted hyenas searching for indications of male-like hormonal conditions.

Results relating to testosterone have been inconsistent. Racey and Skinner (9) and Lindeque *et al.* (10) found no significant differences in plasma testosterone levels of adult male and female spotted hyenas. However, in an extensive study with animals of known social status, Frank *et al.* (11) found the common mammalian pattern: adult males had higher levels of testosterone than did adult females. They further noted that testosterone levels in males were positively correlated with social rank and that the highest ranking female in the social group had testosterone levels six times the female mean, at the median of the male range.

As part of our current developmental study of morphology and behavior in spotted hyenas, we have sampled plasma levels of androgens in males and females during three phases of maturation. We here report data from 20 infants, 25 subadults (prepubertal juveniles over the age of 1 year), and 27 adults, indicating that androstenedione, a precursor of testosterone and estrogen and generally considered a weak androgen, is present in significantly higher concentrations in females than in males and that the sex difference is most prominent in early infancy. Androstenedione exogenously administered during perinatal development has masculinizing effects on adult behavior and genital morphology of rodents (12–15) as well as activational effects in several species (ref. 12, pp. 616–617).

METHODS

Infant spotted hyenas 1–10 weeks of age were collected in Narok District, Kenya, in two cohorts of 10 each during January and December, 1985, and transported to California, where they are maintained in mixed-sex groups of approximately the same age in 400-m^2 enclosures. A cohort of 10 same-age juveniles is typical of the hyena clans in the area from which these originated (16). Ages of infants were determined based on size, stage of tooth eruption, and pelage development (17).

Blood samples were obtained from the jugular vein, following immobilization by intramuscular injection of ketamine hydrochloride (10 mg/kg) and xylazine (1 mg/kg). A random subset of the plasma samples from the wild adults and subadults reported on by Frank *et al.* (11) was assayed for

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androstenedione, as were pre- and postgonadectomy samples from two wild-caught adult females (18). Adults and subadults were aged as in ref. 16. Males were assumed to be sexually mature at the age of 2 years, females at 3 years (2, 3). Ages of the prepubertal animals used in this study were between 6 and 30 months for females and 6 and 12 months for males. All adult females were >3 years of age, whereas the adult males were >2 years old. Finally, two infant females were bilaterally ovariectomized at approximately 4 months of age; the other animals of that cohort were sham-operated. Blood samples were obtained at the time of surgery and 2–5 weeks postoperatively.

Plasma was assayed for testosterone and androstenedione (19). The crossreactivity of the antibodies used for the relevant steroids is as follows: testosterone antibody (androstenedione), 0.007%; androstenedione antibody (testosterone), 0.45%.

RESULTS

Mean plasma testosterone levels are <0.6 ng/ml in all groups except adult males (Fig. 1*A*). Analysis of variance indicates significant effects of sex ($F = 8.84$, df = 1/66, $P < 0.005$), age ($F = 7.92$, df = 2/66, $P < 0.001$), and interaction between sex and age ($F = 5.57$, df = 2/66, $P < 0.01$).

Further examination of data originally analyzed by analysis of variance employed the Dunn–Sidak test for planned multiple "*t*" tests (20). In comparisons between sexes of similar age classes, only in adults are there significant sex differences in testosterone levels ($t = 3.60$, df = 25, $P < 0.01$). Within-sex comparisons across age categories indicate that females have lower testosterone levels during the prepubertal period than as infants ($t = 5.03$, df = 27, $P < 0.01$) or adults ($t = 3.78$, df = 26, $P < 0.01$), whereas age effects in male hyenas are limited to the anticipated increment at puberty ($t = 3.92$, df = 22, $P < 0.01$).

Examination of Fig. 1*B* shows a totally different pattern for androstenedione. Plasma levels are four times greater in female than in male infants. Analysis of variance indicates significant effects of sex ($F = 23.13$, df = 1/66, $P < 0.001$) and age ($F = 10.68$, df = 2/66, $P < 0.001$) but no significant interaction ($F = 2.07$, df = 2/66, not significant). Further analysis shows that male–female differences are significant during the infant ($t = 3.33$, df = 18, $P < 0.05$) and prepubertal ($t = 5.046$, df = 23, $P < 0.01$) periods but not in adults ($t = 2.27$, df = 25, $P > 0.10$). Both males ($t = 3.22$, df = 14, $P < 0.05$) and females ($t = 3.54$, df = 27, $P < 0.01$) exhibit significant decrements in plasma androstenedione levels between the infant and prepubertal periods, but the subsequent increments between prepubertal and adult stages are not statistically significant ($P > 0.10$). Within the infant sample, Fig. 1*C*, the sex difference is even greater if a comparison is made between the four youngest females and the four youngest males (all sampled at 7–8 weeks of age). During this early period, the female mean is more than eight times the male mean ($t = 9.55$, df = 6, $P < 0.01$). However, by the age of 9–14 weeks (using the second monthly plasma sample from the younger infants and the first sample from the older ones), the female androstenedione levels have declined to slightly less than three times the male levels ($t = 3.32$, df = 18, $P < 0.01$).

Higher levels of androstenedione than testosterone were found in every female plasma sample. This also was true for all infant male and 7 of 8 prepubertal male samples. Among adult males, however, testosterone is the more abundant androgen in 10 of 16 animals sampled, and there is a small but significant difference between plasma levels of testosterone and androstenedione ($t = 2.29$, df = 15, $P < 0.05$).

Levels of testosterone and androstenedione are positively correlated *within* age/sex classes, suggesting a common source of these hormones [with the exception of subadult females, in which $r = 0.57$ ($P < 0.05$), all Pearson correlation coefficients ranged between 0.79 and 0.98 ($P < 0.01$)].

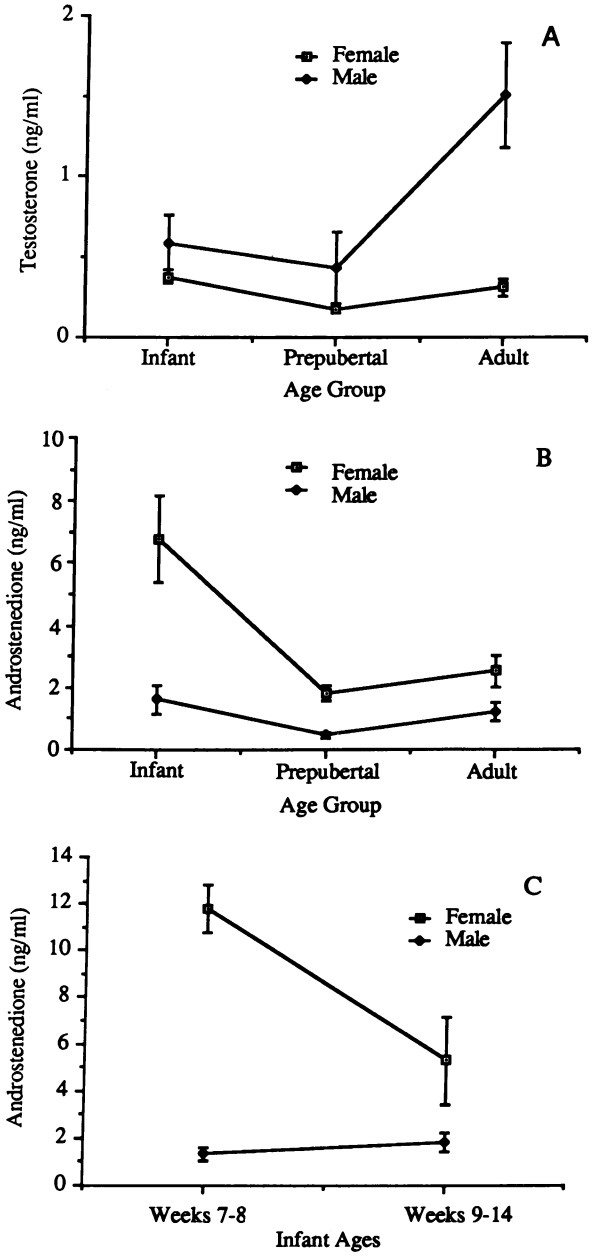

FIG. 1. (*A–C*) Mean (± SEM) testosterone and androstenedione concentrations in spotted hyenas as a function of age and sex. Sample sizes for each age–sex class were as follows: infants = 8 males and 12 females (except the 7- to 8-week-old subjects: 4 males and 4 females); subadults = 8 males and 17 females; adults = 16 males and 11 females.

Ovariectomy resulted in a marked decline in plasma levels of androstenedione in both infant and adult hyenas (Fig. 2). Following sham operation, all four female infants exhibited plasma androstenedione levels of >1 ng/ml, whereas the four ovariectomized females displayed plasma levels of <0.5 ng/ml. An analogous result was obtained with testosterone in our infant hyenas, confirming the prior report of Frank *et al.* (18) based upon adult female hyenas.

DISCUSSION

In 1939, on the basis of histological evidence, Harrison Matthews (3) suggested that the maternal ovary was the

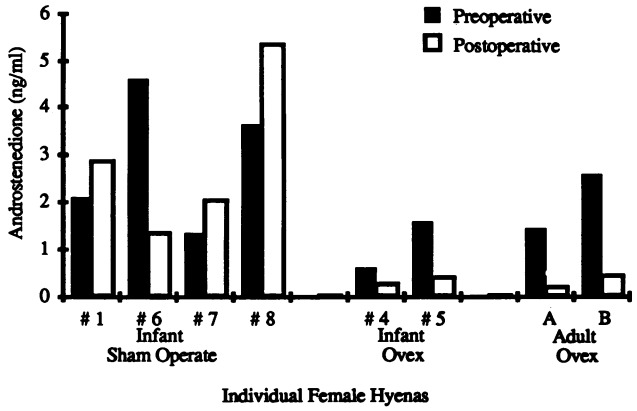

FIG. 2. Effect of ovariectomy (Ovex) on plasma androstenedione levels in female spotted hyenas; controls were sham-operated. Preoperative samples were taken at the time of surgery, and postoperative samples were taken 2–5 weeks afterward.

probable source of androgen responsible for masculinization of the fetal female genitalia in spotted hyenas. Forty years later, Lindeque and Skinner (21) examined androgens in three pregnant hyenas and their fetuses, at estimated fetal ages ranging between 31 and 80 days (the gestation period is estimated at 110 days). Based on discrepancies between the testosterone:androstenedione ratios in maternal and fetal circulations, they ruled out the mother as a source of androgen in the fetus and found no indication of a fetal adrenal contribution. Thus, by elimination, they concluded that the fetal ovary is the source of androgens that masculinize the fetal female hyena. However, in a recent paper, Lindeque *et al.* (10) have reported that in adult hyenas, both the ovaries and adrenals produce androgens following exogenous administration of luteinizing hormone-releasing hormone, human chorionic gonadotropin, or adrenocorticotropic hormone. Data collected by Frank *et al.* (18) from ovariectomized adults and our results from ovariectomized infants suggest that the ovaries are the primary site of androgen production in female spotted hyenas.

Data presented here corroborate the report of Lindeque *et al.* (10), in showing that females consistently have higher levels of androstenedione than do males. Our dataset includes much younger animals, however, and within our infant sample, the largest male–female differential in androstenedione levels appeared in the youngest subjects (Fig. 1*C*). However, data from late second trimester and early third trimester fetuses presented by Lindeque and Skinner show no sex differential in androstenedione. In both sexes, testosterone levels were always higher than androstenedione and there was no instance of the elevated levels of androstenedione seen in our infant female hyenas. Thus, the elevation of androstenedione in developing females may occur during late pregnancy or in the immediate postnatal period.

The ability of the spotted hyena ovary to produce unusually high blood levels of androstenedione, particularly during infancy, may be related to critical developments in the social life of spotted hyenas. For the first month, the two cubs that generally constitute a litter are sequestered in a natal den by the mother. Our observations of three pairs of infants collected during this period show that intense intersibling aggression is characteristic of this early phase of development, consistent with observations of apparent early siblicide among wild-reared infants when both littermates are of the same sex (2). Further intense aggression also apparently occurs when the infants are introduced to the communal den. It seems possible that elevated androstenedione levels in infant females at this age may have organizing or activating effects on aggressive behavior.

Organizational effects in rodents normally occur during pre- or perinatal development. However, Feder (22) has observed that nonrodent mammals may follow somewhat different rules on the timing of the "maximally susceptible period." Beach *et al.* (23) found evidence of organizational effects of androgens in female beagles during an extended period of pre- and postnatal development, and more recently, Ford[¶] has reported that postpubertal administration of exogenous steroids to pigs can organize sexual and aggressive behavior. There is evidence to suggest that genital development in female spotted hyenas is not complete at birth. In at least some female infants the erect phallus is substantially shorter than that of the male but grows to roughly equal size by the age of 3 months (unpublished data). This phase of rapid growth coincides with the period of very high androstenedione levels in the infant female.

In the absence of behavioral and morphological data from female hyenas experimentally deprived of androgenic influences during development, it would be premature to conclude that androstenedione has either organizational or activational effects. However, because of its unique function as a prohormone serving as a precursor of testosterone and estrogen (12), we hypothesize that appropriately timed secretion of androstenedione may promote aggressiveness and masculinize the external genitalia in female hyenas without interfering with normal reproductive function. Neonatal administration of androstenedione to castrated male rats can organize "bisexual" adult behavior, promoting the capacity for displaying male sexual activity without defeminizing the female sexual (i.e., receptive) behavior characteristic of neonatally castrated males (24). The broad gap between male rats and female spotted hyenas notwithstanding, these data provide a plausible model for the peculiar sexual differentiation of *Crocuta*. Because androstenedione is an intermediate product of the mammalian ovary in the biosynthesis of estrogens (25), the adaptation requires only a quantitative change in a known pathway rather than a "new" process. Further studies are needed to assess the ability of various target tissues to form active androgens and estrogens from androstenedione in this species.

While comparisons between free-living and captive subjects must be made with caution, studies of this highly unusual species, when integrated with research on more standard laboratory animals, can provide a significant challenge to our conceptual understanding of the mechanisms underlying sexual differentiation, both anatomical and behavioral. Specifically, this preliminary study suggests that androstenedione's potential as an organizing hormone, capable of producing profoundly male-like characteristics without interfering with essential female function, deserves closer scrutiny in other species. It also calls attention to the potential role of ovarian androgens in sexual differentiation. Although most studies to date have indicated a relatively subtle role (26, 27), further comparative research may well reveal a significant pattern of ovarian influence in other species—e.g., those canids which display intense neonatal aggression (28), and primates such as the spider monkey (*Ateles* spp.), in which females have hypertrophied clitorides (29), and some cercopithecid monkeys—e.g., the talapoin (*Miopithecus talapoin*) and the patas (*Erythrocebus patas*), which display high levels of female aggressiveness (30, 31).

[¶]Ford, J. J., Conference on Reproductive Behavior, June 2–5, 1985, Asilomar, CA, p. 12 (abstr.).

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1. Kruuk, H. (1972) *The Spotted Hyena* (Chicago Univ. Press, Chicago), pp. 1–335.
2. Frank, L. G. (1986) *Anim. Behav.* **35,** 1510–1527.
3. Harrison Matthews, L. (1939) *Philos. Trans. R. Soc. London Ser. B* **230,** 1–78.
4. Neaves, W. B., Griffin, J. E. & Wilson, J. D. (1980) *J. Reprod. Fertil.* **59,** 509–513.
5. Gould, S. J. (1981) *Nat. Hist.* **90,** 16–24.
6. Hamilton, W. J., Tilson, R. L. & Frank, L. G. (1986) *Ethology* **71,** 63–73.
7. Goy, R. W. & McEwen, B. S. (1980) *Sexual Differentiation of the Brain* (MIT Press, Cambridge, MA), pp. 1–222.
8. Jost, A. (1985) in *Handbook of Behavioral Neurobiology*, eds. Adler, N., Pfaff, D. & Goy, R. W. (Plenum, New York), Vol. 7, pp. 3–19.
9. Racey, P. A. & Skinner, J. D. (1979) *J. Zool.* **187,** 315–326.
10. Lindeque, M., Skinner, J. D. & Millar, R. P. (1986) *J. Reprod. Fertil.* **78,** 211–217.
11. Frank, L. G., Davidson, J. M. & Smith, E. R. (1985) *J. Zool.* **206,** 525–531.
12. Whalen, R. E., Yahr, P. & Luttge, W. G. (1985) in *Handbook of Behavioral Neurobiology*, eds. Adler, N., Pfaff, D. & Goy, R. W. (Plenum, New York), Vol. 7, pp. 609–663.
13. Popolow, H. B. & Ward, I. L. (1978) *J. Comp. Physiol. Psychol.* **92,** 13–21.
14. Gilroy, A. F. & Ward, I. L. (1978) *Behav. Biol.* **23,** 243–248.
15. Stern, J. J. (1969) *J. Comp. Physiol. Psychol.* **69,** 608–612.
16. Frank, L. G. (1986) *Anim. Behav.* **35,** 1500–1509.
17. Pournelle, G. H. (1965) *J. Mammal.* **46,** 503.
18. Frank, L. G., Smith, E. R. & Davidson, J. M. (1985) *J. Zool.* **207,** 613–615.
19. Frankel, A. I., Mock, E. J., Wright, W. N. & Kamel, F. (1975) *Steroids* **25,** 73–89.
20. Sokal, R. R. & Rohlf, F. J. (1981) *Biometry* (Freeman, San Francisco).
21. Lindeque, M. & Skinner, J. D. (1982) *J. Reprod. Fertil.* **65,** 405–410.
22. Feder, H. H. (1981) in *Neuroendocrinology of Reproduction: Physiology and Behavior*, ed. Adler, N. (Plenum, New York), pp. 127–158.
23. Beach, F. A., Buehler, M. G. & Dunbar, I. F. (1983) *Behav. Neural Biol.* **38,** 1–31.
24. Goldfoot, D. A., Feder, H. H. & Goy, R. W. (1969) *J. Comp. Physiol. Psychol.* **67,** 41–45.
25. Erickson, G. F., Magoffin, D. A., Dyer, C. A. & Hofeditz, C. (1985) *Endocr. Rev.* **6,** 371–398.
26. Gerall, A. A., Dunlap, J. S. & Hendricks, S. E. (1973) *J. Comp. Physiol. Psychol.* **82,** 449–465.
27. Dunlap, J. L., Gerall, A. A. & Carlton, S. F. (1978) *J. Comp. Physiol. Psychol.* **92,** 280–288.
28. Bekoff, M. (1977) *Am. Nat.* **111,** 715–732.
29. Klein, L. & Klein, D. (1971) *Int. Zoo Yearb.* **11,** 175–181.
30. Dixson, A. F., Scruton, D. M. & Herbert, J. (1975) *J. Zool.* **176,** 177–210.
31. Rowell, T. E. & Chism, J. (1986) *Int. J. Primatol.* **7,** 83–107.